TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2021) 45: 704-716

doi:10.3906/tar-2009-49

Optimization of *Agrobacterium*-mediated transformation and regeneration for CRISPR/Cas9 genome editing of commercial tomato cultivars

Zafer SEÇGİN[1], Musa KAVAS[1,*], Kubilay YILDIRIM[2]

[1]Ondokuz Mayıs University, Faculty of Agriculture, Department of Agricultural Biotechnology, Samsun, Turkey
[2]Ondokuz Mayıs University, Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, Samsun, Turkey

Received: 12.09.2020 • **Accepted/Published Online:** 31.01.2021 • **Final Version:** 16.12.2021

Abstract: Tomato (*Solanum lycopersicum*) is the second most important horticultural crop worldwide that is widely used as a model plant in genetic manipulation of Solanaceae. CRISPR/Cas9 system has been successfully utilized in several studies for genome edition of model tomato cultivars. However, these genome editing systems should be also optimized for commercial tomato cultivar for direct application of genome editing in field conditions. In this study, we have optimized an *Agrobacterium*-mediated gene transfer and regeneration system for CRISPR/Cas9 genome editing in two commercial tomato cultivars for the first time. The effect of explant type, genotype, pre-transformation time, *Agrobacterium* concentration, infection time, and different co-culture periods of bacteria were evaluated to optimize the regeneration and transformation parameters. The highest regeneration capacity of 83% was obtained from cotyledons of Crocker incubated in a medium supplemented with BA (3 mg/L) and IAA (0.1 mg/L). The maximum transformation frequency was obtained by using the following parameters: cotyledon explants of commercial Crocker cultivar that were left for 2 days of pre-transformation incubation, infected with *Agrobacterium* for 10 min at a concentration of OD_{600} of 0.6 and co-cultivated with *Agrobacterium* cells for 48 h. CRISPR/Cas9 system was tested with two gRNAs targeting the *phytoene desaturase* gene. Fully albino and chimeric plants were successfully produced with optimized transformation and culture conditions in up to 71% of all regenerated plants. In the current study, we optimized the implementation of the CRISPR/Cas9 technique in a commercial tomato cultivar and our method will enable breeders to make necessary changes in traits of interest to improve tomato crops for commercial applications.

Key words: CRISPR/Cas9, genome editing, *phytoene desaturase*, plant regeneration, tomato

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the world's major vegetable crops that is widely grown in field and greenhouse conditions in almost every country in the world (Singh et al., 2017). Commercially produced tomatoes are consumed fresh or used to produce tomato-based products. In addition to being a good source of vitamin A, C, K, and potassium, tomatoes have been linked with numerous health benefits due to its rich metabolites such as phytonutrient, lycopene, and carotenoids (Tanambell et al., 2019). Its high consumption and utilization in the food industry caused a stable rise in tomato production, especially in recent years. Tomato has been also used as a model plant to understand the genetic background of fruit quality improvement, plant reproductive enhancement, and plant functional genomics (Khan et al., 2006).

Traditional tomato breeding is generally based on classical hybridization techniques followed by pedigree selection. Backcross breeding has also been used to transfer important traits from wild species to commercial tomato cultivars (Fentik, 2017). Despite important progress in tomato breeding, several important traits related to biotic and abiotic stress tolerance and high fruit quality need to be improved in several cultivars. Due to the recent climate change and global warming, the accelerated introduction of these traits into tomato cultivars became essential in recent years. However, traditional breeding wouldn't allow fast insertion of the desired traits into tomato cultivars due to its time-consuming, untargeted, and laborious nature (Ahmar et al., 2020). On the other hand, plant genome modification through new genome editing tools has progressed greatly in recent years (Čermák et al., 2015; Ma et al., 2015; Jung et al., 2018; Miki et al., 2018; Das Dangol et al., 2019). A novel genome-editing tool called clustered regularly interspaced short palindromic repeats/associated protein 9 (CRISPR/Cas9) has been widely used in recent years to generate genome-edited plants in various species including crops. The discovery of the CRISPR/

* Correspondence: musa.kavas@omu.edu.tr

Cas9 system has made genome editing easier, quicker, cheaper, and accurate (Stukenberg et al., 2018). CRISPR/Cas9 has been successfully used in tomatoes to mutate or edit several functional genes for fruit quality improvement (Čermák et al., 2015; Yu et al., 2017; Deng et al., 2018; X. Li et al., 2018a), fruit crop domestication (Klap et al., 2017; Ueta et al., 2017; Hu et al., 2018; Tomlinson et al., 2019), abiotic stress tolerance (Wang et al., 2017; Yin et al., 2018) and resistance to biotic stresses (Thomazella et al., 2016; Nekrasov et al., 2017; Tashkandi et al., 2018; Zhang et al., 2018; Santillán Martínez et al., 2020). In these CRISPR-mediated genome editing studies, generally, *Agrobacterium*-mediated plant transformation was used only with certain model tomato genotypes. However, regeneration systems and transformation efficiency are highly variable among tomato cultivars and should be optimized before CRISPR/Cas9 application in commercial tomato cultivars.

In the current study, an *Agrobacterium*-mediated CRISPR/Cas9 genome editing system with optimized regeneration was used with two commercial tomato cultivars (Crocker and Bobcat). We selected the *phytoene desaturase* (*PDS*) gene in tomato as a visual marker to test the efficiency of *Agrobacterium*-mediated genome editing of commercial tomato cultivars. These findings will help in the validation and introgression of desirable new traits for tomato crop improvement.

2. Materials and methods

2.1. Plant material and explants preparation

Seeds of tomato (*Solanum lycopersicum*) cv. Crocker and Bobcat were obtained from a commercial seed company (Syngenta). The seeds were surface sterilized by immersion into 70% (v/v) ethanol for 1 min. Then seeds were treated with 20% (v/v) commercial bleach containing tween-20 (0.02 %) for 20 min and rinsed with sterile distilled water five times. The seeds were blot dried on sterile filter paper for half an hour. The surface-sterilized seeds were cultured in a one-liter germination medium (GM) containing glass jars. The pH of all the media was adjusted to 5.7 before autoclaving at 121 °C for 20 min. Plant growth regulators and antibiotics were also added to the media after they were cooled down to 55 °C. Seed germination jars were incubated in the growth chamber for 10 days with a 16/8 h light/dark photoperiod at 25 °C .

2.2. Regeneration of commercial tomato cultivars in tissue culture

Tissue culture and regeneration were firstly optimized for commercial tomato cultivar before *Agrobacterium* transformation and CRISPR-based genome editing. The regeneration potential of commercial tomato cultivars in tissue culture was evaluated under different hormonal combinations. In this context, 6-benzylaminopurine (BAP), kinetin (Kin), and indole-3-acetic acid (IAA) were added into the MS medium (Phytotech labs, KS, USA) in different concentrations with vitamins to optimize the plant regeneration from cotyledon and leaf explants (Table 1). The explants were plated on the respective solid media and transferred onto fresh plates weekly. Each culture was incubated at 26 ± 2 °C with a 16/8h light/dark period. In total, 100 explants (10 explants/plates) were used for each hormonal treatment during the regeneration analysis. The shoot regeneration capacity of leaf and cotyledon explants under different hormonal concentrations was assessed after 4 weeks of culture initiation. Elongated shoots (2–3 cm) were transferred into the root initiation media (RIM) (Table 1). The rooting plants were then transferred to the greenhouse by transplanting them into peat-containing pots.

2.3. Optimization of *Agrobacterium*-mediated transformation parameters in Crocker

Because of the poor regeneration capacity of the Bobcat cultivar, in the current study, we aimed to establish an *Agrobacterium*-mediated transformation system for genome editing of Crocker with the CRISPR/Cas9 system. Since there was no optimized gene transfer protocol for Crocker tomato cultivar, we firstly optimized some transformation parameters including explants type (leaf and cotyledon), bacterial strains (AGL1 and GV3101), bacterial density (OD_{600} 0.3, 0.6, and 0.8), pre-transformation time (1d, 2d, 4d), and co-cultivation duration. Optimization of *Agrobacterium* transformation was carried out using two strains (GV3101 and AGL1) containing the binary CRISPR/Cas9 vector pKI1.1R (without any gRNAs) (Tsutsui and Higashiyama, 2017). *Agrobacterium*-mediated transformation was applied to 10-day-old cotyledons and 30-day-old leaves from seedlings of Crocker cultivar. A single colony of *A. tumefaciens* GV3101 and AGL1 were grown overnight at 28 °C under agitation (220 rpm) in LB medium (25 mL) supplemented with Gentamycin (30mg/L), Rifampicin (10 mg/L), Carbenicillin (50 mg/L), Spectinomycin (100 mg/L) (BGM–Table 1). Overnight cultures of *Agrobacterium* were diluted to the OD_{600} value of 0.3 in IM medium and grown for a further 4–6 h at 28 °C to activate the *vir* genes. *Agrobacterium* cultures were centrifuged, and the pellet was resuspended in 10 mL of inoculation medium (INM) and used to inoculate leaf and cotyledon explants (Table 1). Optimization of INM was carried out with different *Agrobacterium* densities (OD_{600}, 0.1–0.8) grown in two different inoculation media (MS and LB medium). Inoculated explants were blotted on sterile filter paper, transferred into co-cultivating media (CCM - Table 1) and incubated at 25 °C in the dark. After co-cultivation, the infected explants were rinsed two times with washing media (WM). Each inoculated explant was blotted on sterile filter paper and transferred to selective

Table 1. Media and their components used in tissue culture and gene transfer studies in tomato.

Chemicals	GM	PTCM	BGM	IM	INM	CCM	WM	SSRM	RIM
MS (g/L)	4.4	4.4	-	-	4.4	4.4	4.4	4.4	4.4
Luria broth (g/L)	-	-	25	25	25	-	-	-	-
Sucrose (g/L)	20	30	-	-	30	30	30	30	20
Glycine (mg/L)	--	2	-	-	-	2	2	2	-
Myo-Inositol (mg/L)	-	100	-	-	-	100	100	100	-
Nicotinic acid (mg/L)	-	0.50	-	-	-	0.50	0.50	0.50	-
Pyridoxine HCl (mg/L)	-	0.50	-	-	-	0.50	0.50	0.50	-
Thiamine HCl (mg/L)	-	0.10	-	-	-	0.10	0.10	0.10	-
6-BAP (mg/L)	-	3	-	-	-	3	-	3	-
IAA (mg/L)	-	0.1	-	-	-	0.5	-	0.5	0.1
Hygromycin (mg/L)	-	-	-	-	-	-	-	15	25
Cefotaxime (mg/L)	-	-	-	-	-	-	500	500	500
Carbenicilin (mg/L)	-	-	50	50	-	-	-	-	-
Gentamicin (mg/L)	-	-	30	30	-	-	-	-	-
Rifamycin (mg/L)	-	-	10	10	-	-	-	-	-
Spectinomycin (mg/L)	-	-	100	100	-	-	-	-	-
Timentin (mg/L)	-	-	-	-	-	-	80	80	-
Acetosyringone (μM)	-	100	-	200	100	100	-	-	-
Phytagel (g/L)	2.8	2.8	-	-	-	2.8	2.8	2.8	2.8

GM; germination medium, PTCM; pre-transformation culture medium, BGM; bacteria growth media, IM; induction media (activation of vir genes), INM; inoculated media, CCM; co-culture medium, SSRM; selective shoot regeneration medium, and RIM; root initiation medium.

shoot regeneration medium (SSRM) including 15 mg/L hygromycin (Table 1). All explants were subcultured in 7-day intervals and regeneration efficiency was determined by counting the regenerated plants after 4 weeks of agro-inoculation. The transformation efficiency of regenerated plants was verified with PCR amplification of *HptII* and *Cas9* genes (Table 2). For this confirmation, genomic DNA of regenerated T0 tomato plants was extracted from the leaf using the Quick-DNA™ Plant/Seed Miniprep Kit (Zymo Research, CA, USA) following the manufacturer's protocol. The PCR reactions were carried out with Taq 2X Master Mix (New England Biolabs, MA, USA) using the following conditions: initial heat at 95 °C for 3 min followed by 30 cycles consisting of 95 °C for 30 sec, 55 °C for 30 sec, and 68 °C for 60 sec followed by 5 min incubation at 68 °C.

To find the best antibiotic and its concentration that effectively inhibits bacterial growth, explants were also grown on a pre-transformation culture media (PTCM) (Table 1) including the best hormonal concentration selected during tissue culture regeneration tests. Leaf and cotyledons were firstly grown for 1, 2, and 4 days in this PTCM including 3 mg/L BA and 0.1 mg/L IAA (the best hormonal combination for regeneration). Each explant was then inoculated for 10 min with GV3101 and AGL1 *Agrobacterium* strains (OD_{600} of 0.6) carrying binary CRISPR/Cas9 vector. Inoculated explants were transferred into CCM and incubated at three different times (1, 2, or 3 days) at 25 °C in the dark. After co-cultivation, the infected explants were rinsed two times with WM like in the first transformation experiment. Each inoculated explant was then blotted on sterile filter paper and transferred to SSRM having a different concentration of Cefotaxime and Timentin antibiotics. Antibiotic concentration, completely inhibiting the bacterial growth after 2 weeks of Agro-inoculation was selected as the most effective dosage for the next steps.

To decide the best effective concentration of hygromycin during the selection of putative transgenic plants, the cotyledon explants of Crocker was also tested under different hygromycin concentrations (0, 5, 10, 15, 20, 30 mg/L). A total of 4 plates including 10 cotyledon explants were used for each hygromycin dose. The minimum hygromycin dosage that could kill all nontransgenic

Table 2. Primers used in this study.

Primer name	Primer sequence (5' - 3')	Product size (bp)
PDS Tomato Seq F	ACTGTGAAATATCCTTATGGCAGG	506
PDS Tomato Seq R	CCGGAATATCACCTGCACCA	
Hygromycin F	CGAAAAGTTCGACAGCGTC	421
Hygromycin R	GGTGTCGTCCATCACAGTTTG	
Cas9 F	AGACCGTGAAGGTTGTGGAC	569
Cas9 R	TAGTGATCTGCCGTGTCTCG	
gRNA1-F	**ATTG**GCTGTTAACTTGAGAGTCCA	PAM-AGG
gRNA1-R	**AAAC**TGGACTCTCAAGTTAACAGC	
gRNA2-F	**ATTG**GTATTGTCCAGCTCTGGTCT	PAM-TGG
gRNA2-R	**AAAC**AGACCAGAGCTGGACAATAC	

ATTG and **AAAC** were added for cloning with the AarI enzyme.

explants was selected as the optimum concentration to be used for selection in subsequent studies.

2.4. Genome editing in tomato plants with CRISPR/Cas9 system

In the current study, tomato *Phytoene desaturase* was selected as a target gene for the knockout with the CRISPR/Cas9 genome editing system. BLAST analysis against tomato genome in Phytozome (phytozome.jgi.doe.gov) was conducted to identify nucleotide sequences showing homology with *Arabidopsis thaliana PDS3* gene (Qui et al., 2007). A single copy gene here referred to as *SlPDS* (Solyc03g123760.2) was identified and potential gRNAs were determined with Benchling software (www.benchling.com). Two single guide RNAs (Table 2) (sgRNAs) targeting the *SlPDS* gene were selected using the guidelines described by Liang et al., (2016) and minimum free energy prediction of individual gRNAs was carried out in RNAfold WebServer (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi). Microhomology score and out of score were calculated using Microhomology-Predictor (Bae et al., 2014). Then, they were transferred into binary CRISPR/Cas9 vector (pKI1.1R) by following the method of Tsutsui and Higashiyama, (2017). Briefly, 100 µM forward and reverse gRNA primers were phosphorylated with *T4 Polynucleotide Kinase* (New England Biolabs, MA, USA) then incubated at 95 °C for 5 min. The mix was cooled slowly down to room temperature to form a double-stranded fragment with overhangs compatible with *Aar*I (Thermo Fisher Scientific, MA, USA) cutting sites in the vector. This short fragment was then ligated into pKI1.1R by restriction ligation reactions, using *AarI* and *T4 Ligase* (Thermo Fisher Scientific) to generate a full gRNA cassette. The presence of the inserted fragment and stability of the final constructs were confirmed by sequencing. The primers used in this study are shown in Table 2. Two binary vectors carrying gRNA-1 (targeting *PDS*-exon 2) and gRNA2 (targeting *PDS*-exon 3) were transformed only into GV3101 *Agrobacterium* strain due to its high transformation efficiency. Regeneration of genome-edited plants was achieved by using the optimized protocol mentioned above. The genomic DNA of chimeric and fully albino tomato plants was extracted from the leaf using the Quick-DNA™ Plant/Seed Miniprep Kit (Zymo Research, CA, USA) following the manufacturer's protocol. PCR analysis was carried out to determine possible mutation events in T0 tomato plants. For this purpose, Q5® High-Fidelity DNA Polymerase (New England Biolabs, MA, USA) was utilized to amplify exon2 and exon 3 region of *SlPDS* with *SlPDS* specific primers (Table 2). The PCR conditions included denaturation at 98 °C for 30 sec followed by 30 cycles consisting of 98 °C for 15 sec, 63 °C for 30 sec, and 72 °C for 30 sec followed by 5 min incubation at 72 °C. The amplified PCR fragments were then cloned into a TA cloning plasmid (Thermo Fisher Scientific) according to the kit procedure. The mutations were identified by using Sanger sequencing of individual clones in both directions. The obtained nucleotide sequences were compared with the wild-type reference of the *SlPDS* gene using the Blastn at NCBI. Additionally, Synthego's ICE program (https://ice.synthego.com/#/) was used to detect mutations in sequencing files.

https://phytozome.jgi.doe.gov/pz/portal.html [accessed 27 May 2021]
https://benchling.com/ [accessed 27 May 2021]
http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi [accessed 27 May 2021]
https://ice.synthego.com/#/ [accessed 27 May 2021]

3. Results and discussion

3.1. Optimization of tissue culture and regeneration parameters of commercial tomato cultivars

A reliable and efficient regeneration system is the fundamental point in the biotechnological improvement of most plants. Therefore, regeneration parameters of two commercial tomato cultivars (Crocker and Bobcat) were firstly optimized with cotyledon and leaf explants under various combinations of BAP, Kinetin, and IAA (Table 3). Regeneration studies were performed with a total of 100 explants for each treatment and shoot induction and regeneration were evaluated after 4 weeks of culture initiation. The effects of the BA-IAA combination on the leaves of both cultivars were relatively lower than the Kinetin-IAA combination for shoot induction and regeneration. The shoot regeneration capacity from the leaves was increased with the rise in kinetin concentration and reached its highest level in 0.1 mg/L IAA and 4 mg/L kinetin containing medium for Crocker (78%) and Bobcat (66%). Likewise, it was determined that these explants incubated in a medium containing 0.5 mg/L IAA also achieved a very close regeneration rate obtained from the medium containing 0.1 mg/L IAA (Table 3). Overall results of shoot regeneration showed that Crocker's shoot regeneration capacity was much better than Bobcat in almost all hormonal combinations. Additionally, the shoot regeneration capacity of the leaves was much higher than cotyledons for both cultivars. These results clearly indicated the significant role of hormonal composition and genotypes on shoot induction and regeneration. Similar effects of these two variables on shoot regeneration were also reported for several tomato cultivars such as Micro-Tom (Pino et al., 2010; Cruz-Mendívil et al., 2011), Rio Grande (Khoudi et al., 2009; Prihatna et al., 2019), and Pusa Ruby (Sarker et al., 2009). Several other regeneration studies from leaves and cotyledons of tomato cultivars such as Castle Rack (Devi et al., 2008), Pusa Uphar (Kaur and Bansal, 2010), Smart 18 (Kalyani and Rao, 2014), and PKM-1 (Sherkar and Chavan, 2014) have resulted in 65%–94% shoot regeneration capacity with different hormonal

Table 3. Evaluation of auxin and cytokinin effects on shoot formation-level from cotyledon and leaf explants of *S. lycopersicum* cvs. Crocker and Bobcat.

PGRs (mg L-1)			Regeneration from leaf explants (%)		Regeneration from cotyledon explants (%)	
IAA	BA	Kin	Crocker	Bobcat	Crocker	Bobcat
0.1	0.5	0	10	0	12	0
	1.0	0	12	12	33	0
	2.0	0	24	17	76	0
	3.0	0	45	8	**83**	12
	4.0	0	10	0	64	0
	0	0.5	0	0	0	0
	0	1.0	0	0	0	0
	0	2.0	22	13	0	13
	0	3.0	36	17	0	33
	0	4.0	**78**	66	0	12
0.5	0.5	0	0	32	0	32
	1.0	0	9	17	17	12
	2.0	0	12	0	75	0
	3.0	0	18	0	79	0
	4.0	0	16	0	48	0
	0	0.5	0	0	0	0
	0	1.0	10	0	0	0
	0	2.0	32	0	0	0
	0	3.0	40	17	0	13.3
	0	4.0	76	58	0	50

combinations. Our results of 78% and 83% maximum regeneration capacity from leaves and cotyledons, respectively agree with previous studies.

3.2. *Agrobacterium*-mediated transformation of commercial tomato Crocker

In the current study, tomato transformation was optimized by testing several factors such as *Agrobacterium* strain, explant source (leaf and cotyledon), bacterial density, antibiotic concentration, pre-transformation incubation time, and co-cultivation duration.

It has been widely reported that AGL1 and GV3101 were the most efficient strains for the transformation of plants including tomato (Hansen, 2000; Khanna et al., 2007). Therefore, a CRISPR/Cas9 plasmid pKI1.1R (without gRNAs) carrying *HptII* and *Cas9* genes was transformed into AGL1 and GV3101 by electroporation. After colony PCR, both *Agrobacterium* strains were inoculated onto the leaves and cotyledons at different OD_{600} of 0.1, 0.6, and 0.8 to test the survival rates of explants during transformation. The first result of the bacterial test indicated that AGL1 was highly lethal in all tested explants and can cause severe necrosis even at low bacterial densities. Similar to AGL1, GV3101 inoculated leaves of both cultivars also presented severe necrosis within 3 weeks and died regardless of bacterial densities. Fortunately, GV3101 inoculated Crocker cotyledons showed a high survival rate and shoot regeneration capacity after *Agrobacterium* inoculation. Despite the better regeneration response of the leaves in tissue culture, cotyledons were the best explant type for the *Agrobacterium* transformation of tomato Crocker. On the other hand, AGL1 was found to be not suitable for the transformation of tomato leaves and cotyledons. Therefore, Crocker cotyledons and GV3101 Agrobacterium strains were used in subsequent transformation and experiments in the current study. As shown in Figure 1, agro-inoculation of tomato cotyledons with MS inoculation media achieved much better transformation efficiencies compared to LB medium. The bacterial growth in MS medium at OD600 of 0.6 was found to be the most effective concentration for tomato transformation. Previously, several *Agrobacterium* strains have been successfully utilized to create transgenic tomato plants. However, transformation frequencies varied greatly between experiments, explants, and cultivar (Sun et al., 2006; Qiu et al., 2007; Cruz-Mendívil et al., 2011). Chetty et al. (2013) evaluated four *Agrobacterium* strains (AGL1, EHA105, GV3101, and MP90) for the genetic transformation of the Micro-Tom tomato cultivar. Similarly, to our results, the authors found that the highest transformation rate was achieved by GV3101 (65%) followed by EHA105 (40%), AGL1 (35%), and MP90 (15%). Chetty et al. (2013) also reported that the lowest explant mortality rate was observed in GV3101 inoculated cotyledons.

Persistent overgrowth was also a major problem for tomato transformation in the current study. To overcome these problems, two antibiotics (cefotaxime and timentin) inhibiting the growth of *Agrobacterium* were tested in Crocker cotyledon explants transformed with the GV3101 strain. The result showed that cefotaxime (500 mg/L) was more effective than timentin (160 mg/L) for the elimination of bacterial outgrowth. The best bacterial elimination was achieved (5% residual bacterial growth) when 500 mg/L cefotaxime and 80 mg/L timentin were used together (Figure 1). During transformation studies, Crocker cotyledons directly inoculated with Agrobacterium showed tissue browning and loss of viability. Therefore, we optimized the durations of pre-transformation and co-cultivation during tomato transformation. Maximum transformation frequency for Crocker cotyledons was achieved by 2 days of pre-transformation culture followed by 2 days of co-cultivation (Figure 1).

The CRISPR vector utilized in the current study had a *hygromycin resistance* gene as a selection marker. Therefore, the sensitivity of cotyledons to hygromycin dosage (0, 5, 10, 15, 20, 30 mg/L) was also evaluated in a separate experiment. In the hygromycin-free environment, shoot regeneration was observed after 15 days of culture initiation whereas explants cultured on media containing more than 10 mg/L hygromycin presented severe dose-dependent necrosis on the given doses (Figure 2). Based on this investigation, 10 mg/L hygromycin was chosen as the optimum concentration for the selection of transgenic tomato plants.

3.3. CRISPR/Cas9 vector construction and tomato transformation with optimized culture condition

In the current study, we optimized regeneration and *Agrobacterium*-mediated transformation of commercial cultivars of tomato and used optimal conditions to mutate the *Phytoene desaturase* (*SlPDS*) gene using CRISPR/Cas9. *PDS* is responsible for the catalyzing of the conversion of phytoene into a colorful compound in the carotenoid biosynthesis pathway. Inactivation or knock-out of the *PDS* gene disrupts chlorophyll and carotenoid biosynthesis and result in albino and dwarf plants (Tian, 2015; Kaur et al., 2018). Therefore, the *PDS* gene has been used extensively as a molecular and morphological marker for the demonstration of genome editing in several plant species (Hsu et al., 2019; Wilson et al., 2019; Hus et al., 2020) including tomato (Pan et al., 2016; Parkhi et al., 2018).

In the current study, CRISPR/Cas9 mediated disruption of the *SlPDS* gene in tomato was tested with gRNA1 and gRNA2 designed to target exon 2 and exon 3 of the gene, respectively (Figure 3). These gRNAs were selected according to their low off-target capacities and appropriate folding performances with the Cas9 enzyme.

A

Cefotaxime (mg/L)	Timentin (mg/L)	(%) explants with Agrobacterium growth
300	0	80
400	0	35
500	0	20
0	160	85
0	320	28
500	80	5

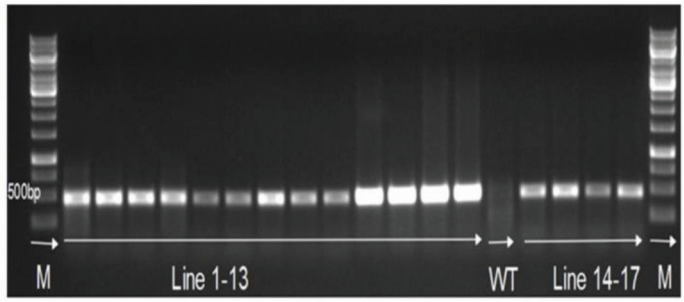
B

Inoculation media	Agrobacterium Cont. (OD600)	Transformation efficiency (%)
MS	0.1	16
MS	0.6	44
MS	0.8	24
LB	0.1	32
LB	0.4	24
LB	0.8	6

C

Pre-culture time (day)	Bacterium infection time (min)	Co-cultivation time (day)	Transformation efficiency (%)
1	10	1	18
1	10	2	24
1	10	4	11
2	10	1	17
2	10	2	42
2	10	4	15
4	10	1	1
4	10	2	4
4	10	4	2

D



E

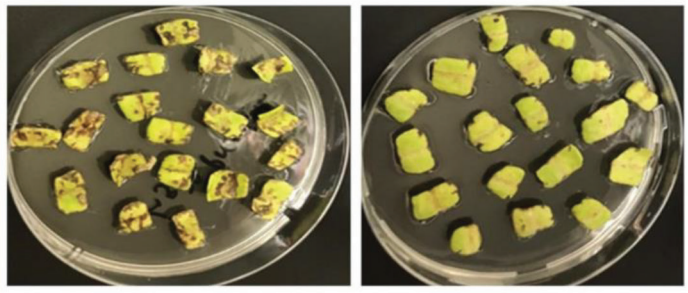
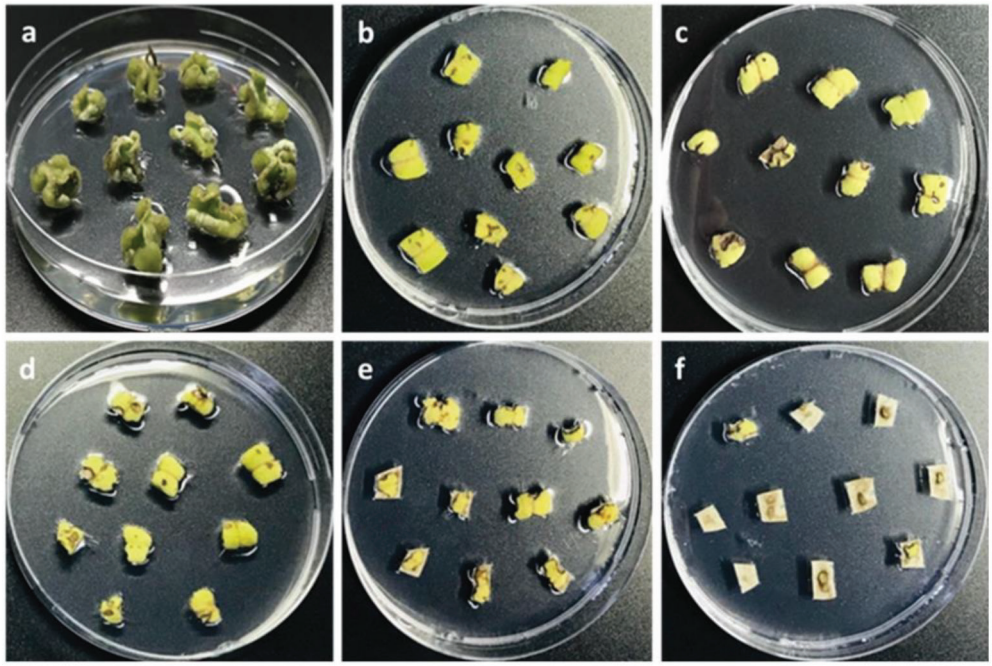


Figure 1. A: indicates the effect of different concentrations of cefotaxime and timentin on the regeneration of tomato explants. B: represents the effect of *Agrobacterium* concentration and inoculation media on transformation efficiency in tomato explants and C: shows the effect of pre-condition and co-cultivation duration on transformation efficiency. D: PCR amplification of *HptII* gene from genomic DNA prepared from each independent line of transgenic plants (T1 line). E: Effect of *Agrobacterium* concentration and inoculation media: a; LB medium OD600 = 0.8, b; MS medium OD600 = 0.8.

The GC content of gRNA1 and gRNA2 were calculated as 45% and 50%, respectively (Figure 4). Both gRNAs were cloned into the plant expression vector pKI1.1R under the control Arabidopsis constitutive U6-26 promoter (Figure 3). In the same vector 2 RPS5A promoter was utilized for expressing of human-codon optimized Cas9 (see Figure 3). The resulting constructs were then transformed into *Agrobacterium* GV3101 due to its high transformation efficiency on Crocker cotyledons and its optimized regeneration protocol mentioned above.

CRISPR-mediated *PDS* mutants appeared after 30 days of explant transformation. In total, 21 transgenic mutant

A



B

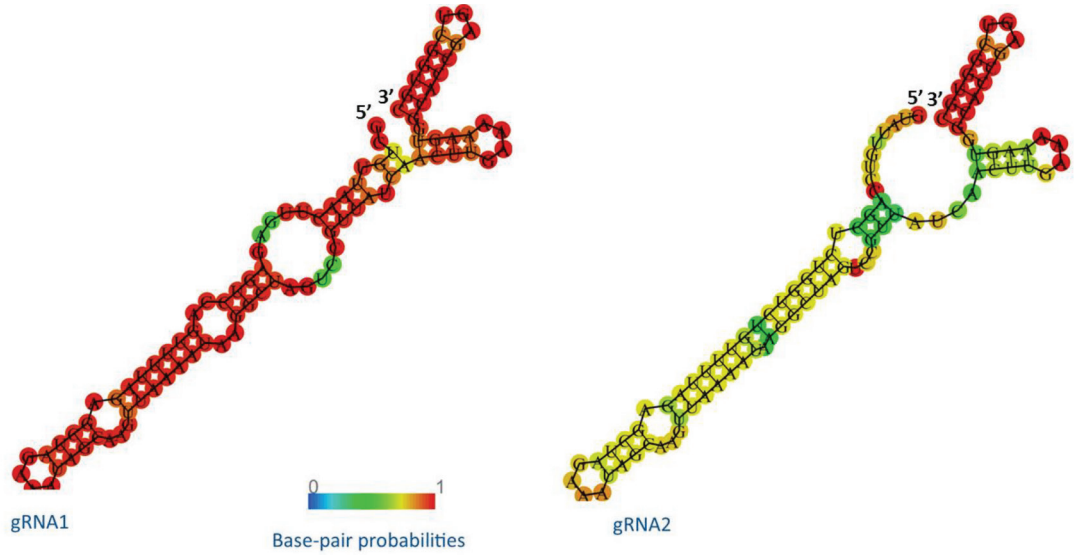
Hygromycin (mg/L)	Explant mortality (%)	Shoot regeneration(%)
0	0	84
5	10	5
10	55	0
15	60	0
20	85	0
30	100	0

Figure 2. A: Effect of hygromycin on plant regeneration of nontransgenic tomato. a: Control, in the absence of hygromycin, b: 5 mg/L hygromycin, c: 10 mg/L hygromycin, d: 15 mg/L hygromycin, e: 20 mg/L hygromycin, f: 30 mg/L hygromycin, respectively inhibited the growth of cotyledon showed necrosis. (15 days). B: Effect of hygromycin on explant mortality and shoot regeneration.

lines were obtained by targeting the exon2 of the *PDS* gene with sgRNA1. Among all the mutant lines, 15 had pure white albino phenotype while 6 transgenic plants had chimeric morphology (Figure 3). lbino and dwarf tomato shoots died within approximately three weeks. Other regenerated shoots (3–5 cm) having chimeric and green phenotypes were transferred into the rooting medium for further analysis. Albino, chimeric and control plants were then used for DNA extraction and PCR amplification to detect the integration of transfer DNA (T-DNA) with hygromycin and Cas9 specific primers. The PCR results confirmed the presence of Cas9 and hygromycin transgenes in all albino and chimeric lines.

Unfortunately, we couldn't obtain any albino and chimeric plants for the second gRNA (gRNA2) targeting the exon 3 of the *PDS* gene. We thought that this might be due to the intrinsic properties of gRNA2 like its secondary structure. As stated in Liang et al., (2016) and some other studies, the GC ratio of designed gRNAs should be between 30% and 80% for effective CRISPR genome editing in plants. This ratio is known to be work by function prerequisite for the gRNAs to work effectively (Liu et al., 2016). However, the GC contents of mutating gRNA1 (45%) and nonmutating gRNA2 (50%) were very close to each other and were compatible with the desired values in previous studies (Liu et al., 2016). The most important parameter that may affect the mutagenesis success is gRNAs on-score values. The on-score values of gRNA1 and gRNA2 are 0.457 and 0.168, respectively. Another important criterion that might affect the mutation rate of gRNAs is base pairing between the selected spacer and scaffold sequence. It is desirable that the total base pairing

A



B

	Microhomology Score	Out-of-frame Score	IBP	TBP	CBP	GSL	TSL	RNA free energy (kcal/mol)	17 N (A/T)	Off-Target	Position	% GC	Micro-Score	On-Score
gRNA1	4733	67.01	0	10	4	0	3	-1.3	T	0	Exon2	45	69.83	0.457
gRNA2	4176	60.32	0	14	8	0	3	-1	G	0	Exon3	50	68.26	0.168

Figure 3. Features of candidate gRNAs**.** A) Schematic representation of the gRNA secondary structure. B) Advanced sgRNA selection process based on the following features: Micro-score: the sum of all patten scores according to the microhomology size and the deletion length. IBP: internal base pairs in the guide sequence. TBP: total base pairs between guide sequence and scaffold. CBP: consecutive base pairs gRNA and scaffold complex. GSL: Stem-loop in the guide sequence (20 nt). TSL: stem-loop in a total of the sgRNA. 17 N(A/T): 17th nucleotide from the 5 ends of the gRNA. GC: guanine and cytosine ratio of gRNA.

(TBP), internal base-pairing (IBP), and consecutive base pairing (CBP) should be less than or equal to 12, 6, and 7, respectively (Liang et al., 2016, Uniyal et al., 2019). In the current study, gRNA1 completely meet all these criteria while gRNA2 was not suitable in terms of CBP (8) and TBP (14) values.

3.4. Molecular confirmation of CRISPR/Cas9-mediated *PDS* mutagenesis in transgenic tomato plants

Three randomly selected albino and one chimeric transgenic tomato plants were selected for mutation confirmation in the *PDS* gene. Genomic DNA was extracted from these plants and *PDS*-exon 2 region (900 bp) containing gRNA1 target was amplified with PCR. All the PCR products were then cloned into a TA cloning vector and plasmid DNA of 4 white colonies were sequenced for each line. Two transgenic albino plant lines were homozygous for mutation on the targeted *PDS* gene with thymine insertion (Figure 5, line E5) and deletion (Figure 5, line E6) at the expected position at 4 bp upstream of the PAM sequence. Interestingly, the third albino line contained a thymine deletion at the 72 bp position upstream of the PAM sequence (Figure 5, line H3). Another interesting result was recorded for the chimeric tomato plant. Some leaves of this chimeric plant were complete albino, while some others showed small white spots. All the white leaf spots and total albino leaves on the chimeric plants turn into a green color after subculturing. Sequencing results of this chimeric genotype indicated that there was a substitution of thymine to cytosine downstream of the PAM sequence (Figure 5, line F12).

In different plant species, the mutation frequency of the CRISPR/Cas9 system is reported to be between 30%–85% (Kaur et al., 2018). Pan et al. (2016) reported a high mutation frequency (83.56%) for *PDS* mutated transgenic 'Micro-Tom' tomato cultivar. In our study, the mutation efficiency was 71% with CRISPR/Cas9 binary vector with a single gRNA targeting exon 2. In addition to *PDS* genes, several tomato genes functional in yield, fruit quality, biotic and abiotic stress tolerance have been successfully targeted and mutated with CRISPR/Cas9 system in tomato (Čermák et al., 2015; Thomazella et al., 2016; Klap et al., 2017; Nekrasov et al., 2017; Ueta et al., 2017; Wang et al., 2017; Yu et al., 2017; Deng et al., 2018; Hu et al., 2018; R. Li et al., 2018b; X. Li et al., 2018a; Tashkandi et al., 2018; Tomlinson

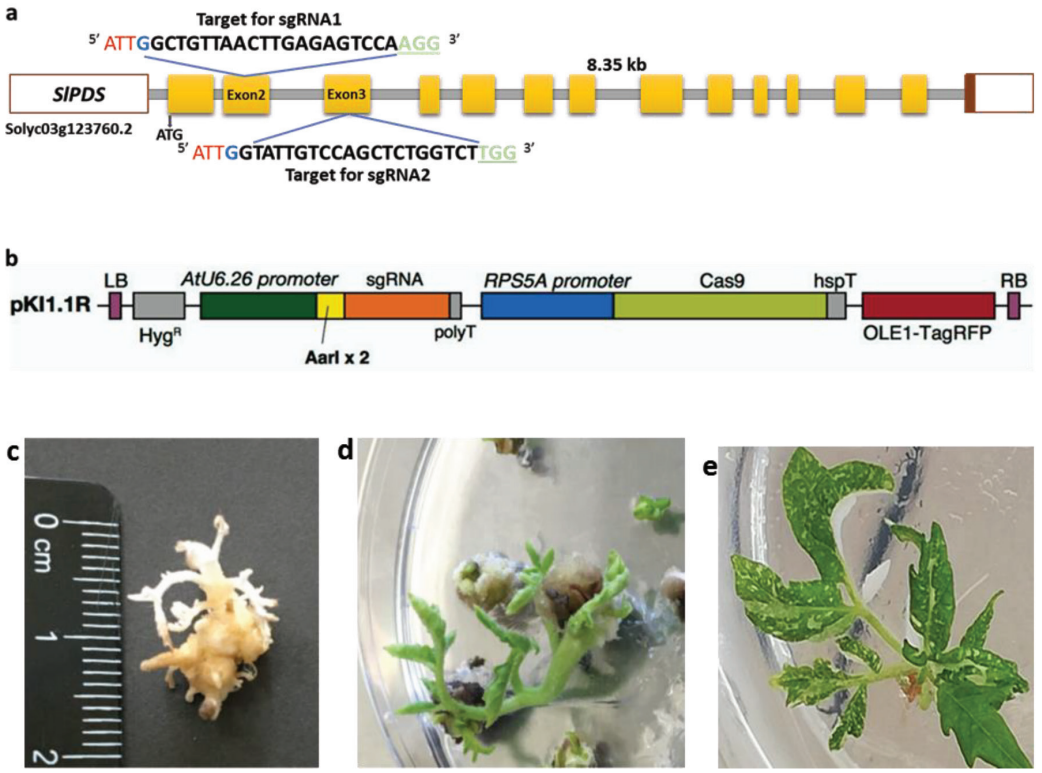


Figure 4. Schematic diagram of the assembled Cas9/sgRNAs expression vector (pKI1.1R) and transgenic tomato line. a) Structural organization of the *SlPDS* gene with its exons and introns. The gRNA sequences designed from the encircled exon2 and exon3 were also represented. b) Schematic representation of the CRISPR/Cas9 binary vector PKI1.1R used for *Agrobacterium*-mediated transformation of tomato. c) transgenic *PDS* mutated albino tomato d) Nonedited wild-type control plants with fully green shoots, e) chimeric albino plant showing a white patch on the leaves and full albino leaves.

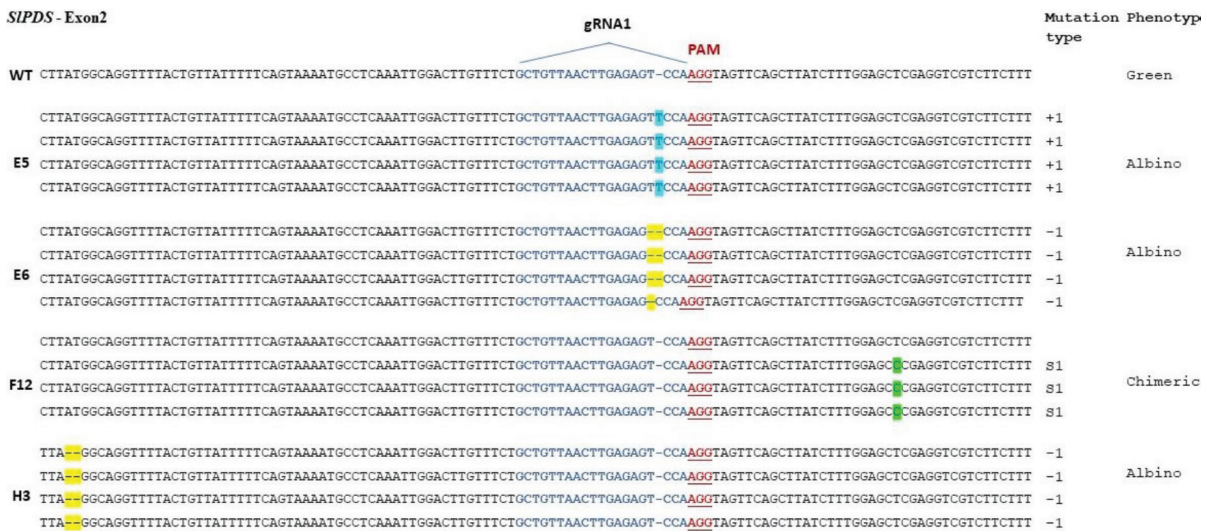


Figure 5. Sequence-based detection of mutations induced by CRISPR/Cas9 constructs *SlPDS* gRNA1. For each plant line characterized, PCR was used to amplify across the target region. The PCR product was cloned into a vector and transformed into *E. coli*. Multiple individual colonies were analyzed via Sanger sequencing to detect mutations near the target site. Aligned sequence data is shown for 4 representative mutant plant lines. The target region of *SlPDS* is in bold and blue in the wild-type (WT) reference sequence, with the protospacer adjacent motif (PAM) in red and underlined. Deletions are highlighted in yellow, insertions are highlighted in light blue, and substitutions are highlighted in green. The mutation type, such as insertion (+), deletion (-), or substitution (S) and size are indicated at the right side of the panel.

et al., 2019; Yin et al., 2018; Zhang et al., 2018; Hus et al., 2020). However, almost all these studies used model tomato cultivars such as Micro-Tom and M-82 (Brooks et al., 2014; Čermák et al., 2015) with limited use for breeders and farmers. We optimized an effective *Agrobacterium*-mediated gene transfer and regeneration system of commercial tomato Crocker cultivar by targeting the *PDS* gene with CRISPR/Cas9 system. Ultimately, our findings provide important improvements in the regeneration and transformation of commercial tomato cultivars and offer an effective utilization of CRISPR/Cas9 genome editing for tomato breeding.

Acknowledgment
This study was supported by the Research Fund of Ondokuz Mayıs University (PYO.ZRT.1901.17.010). We would like to thank Dr. Hiroki Tsutsui for providing the pKI1.1R plasmid.

References

Ahmar S, Gill RA, Jung KH, Faheem A, Qasim MU et al. (2020). Conventional and molecular techniques from simple breeding to speed breeding in crop plants: recent advances and future outlook. International Journal of Molecular Sciences 21 (7): 2590. doi: 10.3390/ijms21072590

Bae S, Kweon J, Kim H, Kim HS, Kim JS (2014). Microhomology-based choice of Cas9 nuclease target sites. Nature Methods 11: 705-706. doi: 10.1038/nmeth.3015

Brooks C, Nekrasov V, Lippman ZB, Van Eck J (2014). Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiology 166 (3): 1292-1297. doi: 10.1104/pp.114.247577

Čermák T, Baltes NJ, Čegan R, Zhang Y, Voytas DF (2015). High-frequency, precise modification of the tomato genome. Genome Biology 16 (1): 232. doi: 10.1186/s13059-015-0796-9

Chetty V, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W et al. (2013). Evaluation of four *Agrobacterium* tumefaciens strains for the genetic transformation of tomato (Solanum lycopersicum L.) cultivar Micro-Tom. Plant Cell Reports 32 (2): 239-247. doi: 10.1007/s00299-012-1358-1

Cruz-Mendívil A, Rivera-López J, Germán-Báez LJ, López-Meyer M, Hernández-Verdugo S et al. (2011). A simple and efficient protocol for plant regeneration and genetic transformation of tomato cv. Micro-Tom from leaf explants. HortScience 46 (12): 1655-1660. doi: 10.21273/HORTSCI.46.12.1655

Devi R, Dhaliwal MS, Kaur A, Gosal SS (2008). Effect of growth regulators on in vitro morphogenic response of tomato. Indian Journal of Biotechnology 7: 526-530.

Dangol D, Barakate S, Stephens J, Çalışkan ME et al. (2019). Genome editing of potato using CRISPR technologies: current development and future prospective. Plant Cell Tissue Organ Cult 139: 403-416. doi: 10.1007/s11240-019-01662-y

Deng L, Wang H, Sun C, Li Q, Jiang H et al. (2018). Efficient generation of pink-fruited tomatoes using CRISPR/Cas9 system. Journal of Genetics and Genomics 45 (1). doi: 10.1016/j.jgg.2017.10.002

Fentik DA (2017). Review on genetics and breeding of tomato (Lycopersicon esculentum Mill). Advanced Crop Science Technology 5 (5): 306. doi: 10.4172/2329-8863.1000306

Hansen G (2000). Evidence for *Agrobacterium*-induced apoptosis in maize cells. Molecular Plant Microbe Interactions 13 (6): 649-657. doi: 10.1094/MPMI.2000.13.6.649

Hsu CT, Cheng YJ, Yuan YH, Hung WF, Cheng QW et al. (2019). Application of Cas12a and nCas9-activation-induced cytidine deaminase for genome editing and as a non-sexual strategy to generate homozygous/multiplex edited plants in the allotetraploid genome of tobacco. Plant Molecular Biology 101: 355-371. doi: 10.1007/s11103-019-00907-w

Hu J, Israeli A, Ori N, Sun TP (2018). DELLA-ARF/IAA interaction mediates crosstalk between gibberellin and auxin signaling in controlling fruit initiation in Solanum lycopersicum. The Plant Cell. doi: 10.1105/tpc.18.00363

Hus K, Betekhtin A, Pinski A, Rojek JM, Grzebelus E et al. (2020). A CRISPR/Cas9-based mutagenesis protocol for Brachypodium distachyon and its allopolyploid relative, Brachypodium hybridum. Frontiers Plant Science 11: 614. doi: 10.3389/fpls.2020.00614

Jung YJ, Nogoy FM, Lee SK, Cho YG, Kang KK (2018) Application of ZFN for site directed mutagenesis of rice SSIVa gene. Biotechnology and Bioprocess Engineering 23 (1): 108-115. doi: 10.1007/s12257-017-0420-9

Kalyani BG, Rao S (2014). Effect of hormones on direct shoot generation in leaf explants of tomato. International Journal of Research in Biotechnology and Biochemistry 4 (1): 20-22. doi: 10.15835/nsb213556.

Kaur N, Alok A, Kaur N, Pandey P, Awasthi P et al. (2018). CRISPR/Cas9-mediated efficient editing in phytoene desaturase (*PDS*) demonstrates precise manipulation in banana cv. Rasthali genome. Functional & Integrative Genomics 18 (1): 89-99. doi: 10.1007/s10142-017-0577-5

Kaur P, Bansal KC (2010). Efficient production of transgenic tomatoes via *Agrobacterium*-mediated transformation. Biologia Plantarum 54 (2): 344-348. doi: 10.1007/s10535-010-0060-9

Khan MS, Usman M, Lilla MI (2006). Facile plant regeneration from tomato leaves induced with spectinomycin. Pakistan Journal of Botany 38 (4): 947.

Khanna HK, Paul JY, Harding RM, Dickman MB, Dale JL (2007). Inhibition of *Agrobacterium*-induced cell death by antiapoptotic gene expression leads to very high transformation efficiency of banana. Molecular Plant Microbe Interaction 20 (9): 1048-1054. doi: 10.1094/MPMI-20-9-1048

Khoudi H, Nouri KA, Gouiaa S, Masmoudi K (2009). Optimization of regeneration and transformation parameters in tomato and improvement of its salinity and drought tolerance. African Journal of Biotechnology 8 (22): 6068-6076. doi: 10.5897/AJB09.057

Klap C, Yeshayahou E, Bolger AM, Arazi T, Gupta SK et al. (2017). Tomato facultative parthenocarpy results from Sl AGAMOUS-LIKE 6 loss of function. Plant Biotechnology Journal 15 (5): 634-647. doi: 10.1111/pbi.12662

Li R, Zhang L, Wang L, Chen L, Zhao R et al. (2018a). Reduction of tomato-plant chilling tolerance by CRISPR–Cas9-mediated SlCBF1 mutagenesis. Journal of Agricultural Food Chemistry 66 (34): 9042-9051. doi: 10.1021/acs.jafc.8b02177

Li X, Wang Y, Chen S, Tian H, Fu D et al. (2018b). Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. Frontiers Plant Science 9: 559. doi: 10.3389/fpls.2018.00559

Liang G, Zhang H, Lou D, Yu D (2016). Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. Scientific Reports 6: 21451. doi: 10.1038/srep21451

Liu X, Homma A, Sayadi J et al. (2016). Sequence features associated with the cleavage efficiency of CRISPR/Cas9 system. Scientific Reports 6: 19675

Ma L, Zhu F, Li Z, Zhang J, Li X et al. (2015). TALEN-based mutagenesis of lipoxygenase LOX3 enhances the storage tolerance of rice (Oryza sativa) seeds. Plos One 10 (12): e0143877. doi: 10.1371/journal.pone.0143877

Miki D, Zhang W, Zeng W, Feng Z, Zhu JK (2018). CRISPR/Cas9-mediated gene targeting in Arabidopsis using sequential transformation. Nature Communications 9 (1): 1-9. doi: 10.1038/s41467-018-04416-0

Nekrasov V, Wang C, Win J, Lanz C, Weigel D et al. (2017). Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. Scientific Reports 7 (1): 1-6. doi: 10.1038/s41598-017-00578-x

Pan C, Ye L, Qin L, Liu X, He Y et al. (2016). CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. Scientific Reports 6: 24765. doi: 10.1038/srep24765

Parkhi V, Bhattacharya A, Choudhary S, Pathak R, Gawade V et al. (2018). Demonstration of CRISPR-cas9-mediated pds gene editing in a tomato hybrid parental line. Indian Journal of Genetics and Plant Breeding 78 (1): 132-137. doi: 10.5958/0975-6906.2018.00016.0

Pino LE, Lombardi CS, Azevedo MS, Scotton DC, Borgo L et al. (2010). The Rg1 allele as a valuable tool for genetic transformation of the tomato 'Micro-Tom' model system. Plant Methods 6 (1): 23. doi: 10.1186/1746-4811-6-23

Prihatna C, Chen R, Barbetti MJ, Barker SJ (2019). Optimisation of regeneration parameters improves transformation efficiency of recalcitrant tomato. Plant Cell, Tissue and Organ Culture 137 (3): 473-483. doi: 10.1007/s11240-019-01583-w

Qiu D, Diretto G, Tavarza R, Giuliano G (2007). Improved protocol for *Agrobacterium* mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene CsZCD. Scientia Horticulturae 112 (2): 172-175. doi: 10.1016/j.scienta.2006.12.015

Santillán Martínez MI, Bracuto V, Koseoglou E. Appiano M, Jacobsen E et al. (2020). CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene PMR4 for resistance against powdery mildew. BMC Plant Biology 20: 284. doi: 10.1186/s12870-020-02497-y

Sarker RH, Islam K, Hoque M (2009). In vitro regeneration and *Agrobacterium*-mediated genetic transformation of tomato (Lycopersicon esculentum Mill.). Plant Tissue Culture and Biotechnology 19 (1): 101-111. doi: 10.3329/ptcb.v19i1.5004

Sherkar HD, Chavan AM (2014). Studies on callus induction and shoot regeneration in tomato. Science Research Reporter 4 (1): 89-93.

Sing H, Kumar P, Chaudhari S, Edelstein M (2017). Tomato grafting: a global perspective. HortScience 52 (10): 1328-1336. doi: 10.21273/HORTSCI11996-17

Stukenberg D, Zauner S, Dell'Aquila G, Maier UG (2018). Optimizing CRISPR/Cas9 for the diatom Phaeodactylum tricornutum. Frontiers in Plant Science 9: 740. doi: 10.3389/fpls.2018.00740

Sun HJ, Uchii S, Watanabe S, Ezura H (2006). A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiology 47 (3): 426-431. doi: 10.1093/pcp/pci251

Tanambell H, Quek SY, Bishop KS (2019). Screening of in vitro health benefits of tangerine tomatoes. Antioxidants 8 (7): 230. doi: 10.3390/antiox8070230

Tashkandi M, Ali Z, Aljedaani F, Shami A, Mahfouz MM (2018). Engineering resistance against tomato yellow leaf curl virus via the CRISPR/Cas9 system in tomato. Plant Signaling Behavior 13 (10): e1525996. doi: 10.1080/15592324.2018.1525996

Thomazella D, Brail Q, Dahlbeck D, Staskawicz B (2016). CRISPR-Cas9 mediated mutagenesis of a DMR6 ortholog in tomato confers broad-spectrum disease resistance. BioRxiv, 064824. doi: 10.1101/064824

Tian L (2015). Recent advances in understanding carotenoid-derived signaling molecules in regulating plant growth and development. Frontiers Plant Science 6: 790. doi: 10.3389/fpls.2015.00790

Tomlinson L, Yang Y, Emenecker R, Smoker M, Taylor J et al. (2019). Using CRISPR/Cas9 genome editing in tomato to create a gibberellin- responsive dominant dwarf DELLA allele. Plant Biotechnology Journal 17 (1): 132-140. doi: 10.1111/pbi.12952

Tsutsui H, Higashiyama T (2017). pKAMA-ITACHI vectors for highly efficient CRISPR/Cas9-Mediated gene knockout in Arabidopsis thaliana. Plant Cell Physiology 58 (1): 46-56. doi: 10.1093/pcp/pcw191

Ueta R, Abe C, Watanabe T, Sugano SS, Ishihara R et al. (2017). Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. Scientific Reports 7 (1): 1-8. doi: 10.1038/s41598-017-00501-4

Uniyal AP, Mansotra K, Yadav SK, Kumar V (2019). An overview of designing and selection of sgRNAs for precise genome editing by the CRISPR-Cas9 system in plants. 3 Biotech 9, 223. doi: 10.1007/s13205-019-1760-2

Wang L, Chen L, Li R, Zhao R, Yang M et al. (2017). Reduced drought tolerance by CRISPR/Cas9-mediated SlMAPK3 mutagenesis in tomato plants. Journal of Agricultural Food Chemistry 65 (39): 8674-8682. doi: 10.1021/acs.jafc.7b02745

Wilson FM, Harrison K, Armitage AD, Simkin AJ, Harrison RJ (2019). CRISPR/Cas9-mediated mutagenesis of phytoene desaturase in diploid and octoploid strawberry. Plant Methods 15 (1): 45. doi: 10.1186/s13007-019-0428-6

Yin Y, Qin K, Song X, Zhang Q, Zhou Y et al. (2018). BZR1 transcription factor regulates heat stress tolerance through FERONIA receptor-like kinase-mediated reactive oxygen species signaling in tomato. Plant Cell Physiology 59 (11): 2239-2254. doi: 10.1093/pcp/pcy146

Yu Qh, Wang B, Li N, Tang Y, Yang S et al. (2017). CRISPR/Cas9-induced targeted mutagenesis and gene replacement to generate long-shelf life tomato lines. Scientific Reports 7 (1): 1-9. doi: 10.1038/s41598-017-12262-1

Zhang S, Wang L, Zhao R, Yu W, Li R et al. (2018). Knockout of SlMAPK3 reduced disease resistance to Botrytis cinerea in tomato plants. Journal of Agricultural and Food Chemistry 66 (34): 8949-8956. doi: 10.1021/acs.jafc.8b02191